EXHIBIT S

TO DECLARATION OF SCOTT D. TANNER, PHD.

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TO DECLARATION OF SCOTT D. TURNER, PHD.

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Review

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Speciation of protein-bound trace elements by gel electrophoresis and atomic spectrometry

The metabolism of trace elements, in particular their binding to proteins in biological systems is of great importance in biochemical, toxicological, and pharmacological studies. As a result there has been a sustained interest over the last two decades in the speciation of protein-bound metals. Various analytical approaches have been employed, combining efficient separation of metalloproteins by liquid chromatography or electrophoresis with high-sensitivity elemental detection. Slab-gel electrophoresis (GE) is a key platform for high-resolution protein separation, and has been combined with autoradiography and various atomic spectrometric techniques for in-gel determination of protein-bound metals. Recently, the combination of GE with state-of-the-art inductively coupled plasma-mass spectrometry (ICP-MS), particularly when linked to laser ablation (LA) for direct gel interrogation, has opened up new opportunities for rapid characterization of metalloproteins. The use of GE and atomic spectrometry for the speciation of protein-bound trace elements is reviewed in this paper. Technical requirements for gel electrophoresis/atomic spectrometric measurement are considered in terms of method compatibilities, detection capability and potential usefulness. The literature is also surveyed to illustrate current status and future trends.

Keywords: Atomic spectrometry / Gel electrophoresis / Protein binding / Review / Trace elements
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Contents

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Abbreviations: AAS, atomic absorption spectrometry; **DRC**, dynamic reaction cell; **ETV**, electrothermal vaporization; **ICP-MS**, inductively coupled plasma-mass spectrometry; **LA**, laser ablation; **PIXE**, proton induced X-ray emission; **SR-XRF**, synchrotron radiation X-ray fluorescence

1 Introduction

Trace elements play a vital role in biochemical processes, being essential components of molecular structure and hence involved in fundamental processes such as catalysis, enzyme regulation, and gene expression. Occupational exposure to metals, chemical pollution, diet and drug treatments based on metallopharmaceuticals can adversely affect metal-protein homeostasis, inducing toxic effects hence the need for robust speciation technologies that combine high separatory power with high detection sensitivity. Strategies for chemical speciation have been principally based on combining separation schemes such as liquid chromatography and electrophoresis, with sensitive atomic spectrometric techniques. For instance much attention has been focused on the development of rapid on-line hyphenated systems that link liquid chromatography and atomic spectrometry. Szpunar [1] has comprehensively reviewed the field of bioinorganic speciation analysis using hyphenated analytical systems. Articles on characterization of metal-binding proteins by high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are also available [2, 3].

Slab-gel electrophoresis (GE) is the benchmark for high-resolution protein separation, particularly in two-dimensional (2-D) format as documented in a wide range of pro-

teomic studies [4, 5]. Electrophoresis under either native or denaturing conditions can be readily implemented according to the nature of the metalloprotein complexes. In contrast to solution-based approaches *via* HPLC and CE, the full potential of GE for metalloprotein speciation has yet to be fully realized. Conventional techniques for gel analysis are typically based on staining of the protein moiety [6, 7] but such approaches provide no specific information on metal ions or heteroatoms. Autoradiography involving radiotracers or neutron activation has traditionally been used for in-gel detection and quantitation of separated metalloproteins. Excellent sensitivity and resolution are achieved with significantly reduced processing time when using modern particle counting detection systems. Cornelis [8], Behne *et al.* [9], and Stone *et al.* [10–12] have outlined the applications of radiotracers or instrumental neutron activation analysis with autoradiography for GE-based metalloproteins speciation. Nevertheless, inconvenience due to radiation hazard, radioactive waste disposal and nuclear facilities limit widespread use of the technique.

Over the past quarter century, atomic spectrometry has been utilized for in-gel elemental analysis as represented by Fig. 1. Earliest studies were based on atomic absorption spectrometry (AAS), X-ray methods, and nuclear techniques, but a recent trend is the increasing use of inductively coupled plasma-mass spectrometry (ICP-MS) [13]. Overall there are some 50 publications in this specialized area of GE-atomic spectrometry. Sample types encompass biological, botanical, and environmental materials and elements of interest include Au, Cd, Co, Cu, Fe, Ga, Hg, In, Ni, P, Pt, S, Se, and Zn.

The aim of this paper is to highlight the use and scope of atomic spectrometry in combination with GE for speciation of protein-bound trace elements in biological systems. Some technical considerations for gel electrophoretic separation of metalloproteins are discussed and atomic spectrometric methodologies are compared with respect to compatibility with GE and detection capability. A survey of current literature is presented to indicate capabilities and current status for the field.

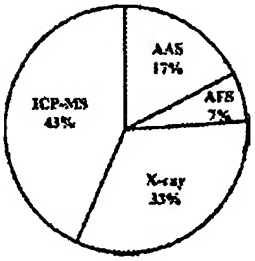

Figure 1. Metalloprotein speciation studies by GE with atomic spectrometric detection.

2 Gel electrophoretic separation of metalloproteins

GE in various formats is extensively used for protein separation in biological systems. One-dimensional (1-D) GE is the simplest format but, in general, cannot provide adequate resolution for complex matrices. The superior resolving power of 2-D gel electrophoresis (2-DE), which can separate thousands of protein species in a proteome [4, 5], represents the state-of-the-art. In the context of metalloprotein separation/speciation, the following formats have been utilized: 1-D polyacrylamide gel electrophoresis (PAGE) under both native and denaturing conditions, slab-gel isoelectric focusing (IEF), 2-DE (IEF plus PAGE), and 2-D crossed immunoelectrophoresis (IEP) on agarose gel. To facilitate detection, whole gel elution and Western blotting of protein bands are frequently practised.

With reference to metalloprotein speciation, a key requirement is to maintain the integrity of metal-protein binding during electrophoresis. Metalloprotein complexes may have limited thermodynamic stability and therefore should be studied under native rather than denaturing conditions [14, 15]. This aspect is well illustrated in Fig. 2, in the case of Pt-serum binding proteins, for native and sodium dodecyl sulfate (SDS) gels which are subject to laser ablation (LA)-ICP-MS [15]. On the other hand, seleno- and phospho-proteins are perfectly compatible with the denaturing processes, as Se and P are covalently bound. Metal-protein bonds may be disrupted when IEF separation is performed on a vertical pH-gradient gel. In

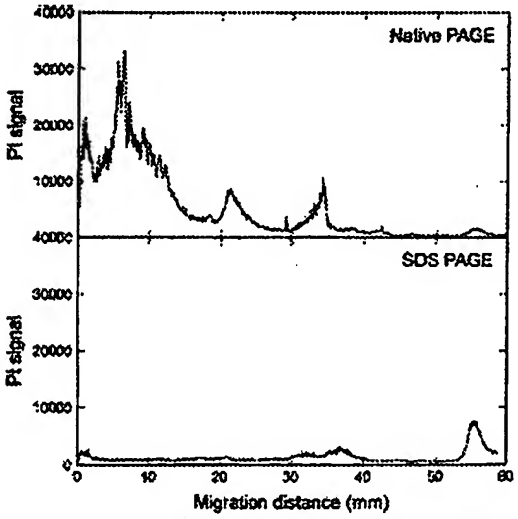

Figure 2. PAGE and SDS-PAGE/LA-ICP-MS (^{195}Pt) for Pt-enriched blood serum.

that case, samples are loaded at the top of the gel where metalloproteins of interest are exposed to a pH regime several units away from their isoelectric points. In flatbed (horizontal) IEF systems, samples are loaded in the center of the gel with a pH value near neutral. Lustig *et al.* [16] found that the weak native V-transferrin complex (p*I* 5.9) in human serum was disrupted when the sample was applied at pH 4.7 for IEF, and released V ions were partially bound to albumin (p*I* 4.8). A weak V-transferrin band could be observed without binding to albumin only when the sample was applied at neutral pH.

A practical problem with commercially available GE systems lies in the fact that they are not intended to meet the requirements of trace/ultratrace element analysis and so potentially may pose a trace element leaching/contamination hazard. The same applies to all the reagents and buffers used in gel processing. Platinum electrodes are universally featured in GE units and consequently Pt contamination of flatbed gels has been reported [16], the Pt concentration level far exceeding the analyte concentration. In one study on Pt speciation in blood serum the Pt electrodes had to be replaced with ultrapure Au electrodes [16]. In a vertical GE system, the gel comes into very little contact with the electrodes, usually thin Pt wire, hence a much reduced Pt background [15].

Sulphur is a natural heteroatom in proteins and extremely useful as a marker of proteins to demonstrate metal-protein association/binding. The element, however, is at hundreds of ppm in polyacrylamide gels (due to use of ammonium persulfate as a catalyst for the acrylamide polymerization) and hence the measurement of S in protein bands is compromised [17]. Also the use of SDS for denaturation precludes S measurement in SDS-based separations. Several studies on phosphoprotein analysis based on direct P measurement by LA-ICP-MS have been hindered by the presence of inorganic phosphate in the gels and reagents [18–20]. Transfer of the protein bands/spots onto relatively clean membranes may minimize this problem [18, 19]. Further washing of the membrane strips with $Ga(NO_3)_3$ was found to be effective for removing inorganic phosphate attached to the protein bands [19]. In general, the presence of trace element impurities in gels and associated reagents is a potential concern in all metalloprotein investigations.

3 Atomic spectrometric detection

Traditional methods for protein detection in gels involve the use of Coomassie blue or silver staining preparations [6]. Recently, the availability of fluorescent stains has resulted in an improved detection capability [7]. However, staining approaches lack specificity with respect to elemental detection. For metalloprotein applications, atomic spectrometry offers high element specificity together with a range of sensitivities depending on the specific technique utilized. The main atomic spectrometric techniques that have been exploited in gel analysis include AAS, atomic fluorescence spectrometry (AFS), X-ray spectrometry and ICP-MS. AAS, AFS, and ICP-MS are primarily designed for liquid sample analysis, whereby sample introduction is achieved by nebulization, electrothermal vaporisation (ETV) or chemical vapor generation. Thus, analytes in gels have first to be solubilized either by acid digestion or electroelution prior to measurement. ETV and LA devices when interfaced to the above sources can be used for direct gel analysis, *i.e.*, without the need for gel solubilization. For analysis involving sample dissolution and ETV (with AAS or ICP-MS), gels may be cut into sections of a selected size or bands/spots are excised following visualisation *via* staining. Alternatively whole-gel elution can yield a number of fractions corresponding to fixed migration-distance intervals along a 1-D gel. These procedures are relatively time-consuming and the additional steps may compromise analysis either from a resolution or a contamination standpoint. The LA technique, in contrast, can provide direct *in situ* interrogation of gels resulting in rapid and efficient transfer of gel contents in fine aerosol form to the ICP-MS. Depending on laser operating parameters and analysis mode, the gel can be interrogated in such a way as to reveal ion intensities for individual protein bands/spots or gel locations (1-D or 2-D). Moreover calibration is readily achieved by casting standard gels enriched with analyte elements [13, 15, 17], hydrating dry gels with aqueous standard solutions [21], or loading gels with standard metalloproteins [19, 22]. Carbon can be used as a natural internal standard, based on the minor isotope ^{13}C, to compensate for variations in LA/ICP-MS response [17, 21]. Calibration by aspirating standard solution (ultrasonic nebulizer) through the LA chamber has also been reported [20].

AAS was the first technique to be used for elemental determinations in gels with electrothermal (ET) atomisation being preferred to flame atomization given the need for microsampling and high sensitivity. Conventional X-ray fluorescence (XRF) has been described for gel analysis but has seen very little uptake due presumably to lack of sensitivity. High-sensitivity particle/proton induced X-ray emission (PIXE) and synchrotron radiation X-ray fluorescence (SR-XRF), although of limited availability for routine applications, are of interest in gel analysis given their multielement capability, good detection limits ($\mu g \cdot g^{-1}$ range) and spatial resolution (mm scale) [23]. ICP-MS, however, is highly competitive given its enhanced sensitivity, wide dynamic range, multielement capability and elemental/isotopic information [24]. More-

over, the use of a high power laser (typically Nd:YAG) for *in situ* microsampling provides a unique interface for GE and ICP-MS [25]. For elements of biological importance, detection limits in the range of $ng \cdot g^{-1}$ to $\mu g \cdot g^{-1}$ may be realized for gel analysis *via* LA-ICP-MS depending on laser type, operating conditions, contamination level, spectral interference, first ionization potential, and abundance of the isotope selected [21].

4 Metalloprotein speciation studies

4.1 Atomic absorption spectrometry

Flatbed native-PAGE was shown by Dunemann *et al.* [26, 27] to be a preferred technique to gel chromatography and flow-through electrophoresis for the separation of metal-protein complexes in soybean flour extracts. Following gel electroelution, flame AAS was used to establish distribution profiles for Cu, Ni, and Zn. ET-AAS has been utilized at an early stage to determine metals in protein bands typically after gel dissolution. Cambar *et al.* [28] studied mineralization conditions for the determination of Hg bound to serum albumin fixed in polyacrylamide gels. A complete destruction of the gel matrix with a mixture of HNO_3-H_2SO_4-$KMnO_4$ was required for quantitative recovery of the complex. For clinical purposes Cu [29, 30] and Au [31] in human serum protein fractions obtained from cellulose acetate membrane electrophoresis were monitored by ET-AAS. Co-binding proteins in serum, plasma, and whole blood from workers occupationally exposed to Co were studied *via* preparative IEF [32]. The Co concentrations in isolated fractions were measured by ET-AAS. In serum and plasma, Co was found only in the fraction corresponding to pH 5. The whole blood samples exhibited signals of differing intensities at pH 5 and 7, and the relative magnitude of signals differed for each sample.

Selenium is an element currently receiving much attention in speciation research. Thus, ET-AAS has been deployed for the direct determination of selenoproteins in polyvinylidene difluoride (PVDF) blotting membranes [33]. The proteins were separated by SDS-PAGE and then quantitatively transferred onto a PVDF membrane by semidry electroblotting. After staining, the protein bands were excised and manually introduced into the graphite furnace. Chemical modifier was added on top of the membrane prior to measurement and no matrix interferences were observed. Signal linearity was achieved in the range of 2–10 ng Se. This labor-intensive method was used for identification of selenoprotein P enriched from human plasma.

4.2 Atomic fluorescence spectrometry

Hydride generation (HG)-AFS detection was developed for SDS-PAGE speciation of selenoproteins in subcellular fractions of human liver [34]. Stained protein bands were removed and digested with a mixture of HNO_3-$HClO_4$ (3:1) at 100°C. Selenium(VI) was reduced to Se(IV) by 5 M HCl prior to reaction with $NaBH_4$. Of 24 protein species detected, the molecular masses were mostly in the ranges of 20–30 kDa and 50–80 kDa. Major Se-containing protein fractions at 61 kDa and 21 kDa were probably selenoprotein P and glutathione peroxidase, respectively. The protein at 54 kDa was probably a thioredoxin reductase, which is present in nuclei, mitochondria, lysosome, microsome, and cytosol. The Se-containing protein with the lowest molecular mass of 9.3 kDa only existed in lysosome. Better protein resolution and molecular weight accuracy were achieved by SDS-PAGE in comparison with gel filtration chromatography, where only eight Se-containing proteins were identified [35].

A similar procedure was described more than a decade earlier by Vezina *et al.* [36] to determine Se in protein bands in polyacrylamide gels and on nitrocellulose blotting membranes through formation of a fluorescent complex with diaminonaphthalene after HNO_3-$HClO_4$ digestion and HCl reduction. A detection limit of 0.06 ng Se and quantitative recoveries of Se in glutathione peroxidase were achieved. In a recent study, Se was determined in four protein bands in human serum after SDS-PAGE [37].

4.3 X-ray spectrometry

Stone *et al.* [12] evaluated bench-top microbeam X-ray spectrometry and energy-dispersive XRF analysis for Se detection in glutathione peroxidase after PAGE. Detection limits of Se in the gel matrix were 2.1 ng for the microbeam X-ray system and 30–60 ng using energy-dispersive XRF. PIXE analysis of metalloproteins separated by SDS-PAGE was first described by Szokefalvi-Nagy and co-workers [38–42]. For detection the gel was dried and sections were scanned in 0.5 mm steps by a collimated 3 MeV proton beam. Gels were stained after analysis to avoid possible loss of analyte or contamination, and correlation between the X-ray spectra and the protein bands was made. In an early study, the Fe content of a hydrogenase enzyme, high-potential iron-sulphur protein (HiPIP, containing 4 Fe-S clusters) from the purple sulphur photosynthetic bacteria *Thiocapsa roseopersicina* was measured [38, 40]. Protein bands containing < 1 µg HiPIP protein were readily detected with the integrated Fe con-

tent being proportional to the amount of protein in the band. In a further study, the enzyme was analyzed to provide relative amounts of Fe and Ni [39, 40].

Quantitative measurement of metals in proteins by PIXE after PAGE separation has also been reported by Weber *et al.* [43–45] using a 2.5 MeV proton beam. For metal quantitation, the characteristic X-ray peak area was compared with signals derived from metal-doped gels. The metal/protein ratios were computed after the relative concentration of each protein was determined by densitometry. Solis *et al.* [46] used PIXE with an external proton beam of 3.7 MeV to characterize Zn enzymes including carbonic anhydrase and cytoplasmic pyrophosphatase of *Rhodospirillum rubrum*, purified by native PAGE.

Homma-Takeda *et al.* [47] combined SR-XRF analysis with IEF to investigate Hg interaction with metallothionein-II (MT-II) and Cu,Zn-superoxide dismutase (Cu,Zn-SOD). When MT-II reacted with mercuric chloride, a clear shift in isoelectric point was observed. This marked migration of MT-II by Hg could be blocked by addition of glutathione, suggesting that sulfhydryl functions participate in the p*I* variation. In contrast, interaction of Cu,Zn-SOD with Hg did not cause any changes of p*I* although Hg was bound tightly to Cu,Zn-SOD after electrophoresis; however, enzyme activity was drastically suppressed.

Gao *et al.* [48] utilized SR-XRF to determine trace elements in metalloproteins of human liver cytosol separated with thin-layer IEF following gel filtration chromatography. The gel was moved at 0.4–1 mm intervals for scanning purposes (0.5 × 0.1 mm beam). In the molecular mass range 10–25 kDa, numerous bands with p*I* values between 4.4 and 7.2 containing Fe (11 species), Zn (2 species), and Cu (2 species) were detected. The study was also performed with SDS-PAGE [49, 50]. In later work the detection of metalloproteins was greatly improved by drying the gel before SR-XRF determination as the continuous background resulting mainly from the Compton-scattering of X-rays by the gel matrix was substantially reduced [50]. Of at least four Fe-containing proteins (20, 23, 43, and 83.5 kDa) reported, two (20 and 23 kDa) were probably two subunits of ferritin broken up during sample pretreatment and SDS-PAGE. Six Zn-containing bands were observed (17.5, 20.5, 27, 35, 55, and 63 kDa) and Cu was present in the 17.5 kDa Zn-containing band, suggesting the protein was Cu,Zn-SOD. The metal contents in dominant bands, such as Fe (23 and 83.5 kDa), Zn (63 kDa), and Cu (22 kDa), were closely related to the metal levels in the human liver cytosol samples. The procedure was verified with a mixture of known metalloproteins: lactate dehydrogenase (high Zn), cytochrome *c*, hemoglobin and transferrin (high Fe), and bovine serum albumin (low Zn, Fe and Cu).

4.4 ICP-MS

A number of research groups have been active in developing ICP-MS-based methodologies for gel analysis. Lustig *et al.* [14] described 2-D IEF/native PAGE for the separation of Pt in serum proteins. Gels were run in duplicate with one being silver-stained for protein detection and the other cut into segments (5 × 20 mm) and digested with *Aqua regia* for determination of Pt. The same approach was subsequently applied to speciate Ga and In in rabbit serum (*in vivo*) [51], where both elements were bound exclusively to transferrin. ETV-ICP-MS has been used for direct analysis of gel [52]. Gel matrix components were separated from analytes of interest *via* a multistage temperature program prior to high-temperature atomisation/vaporization of Se. Chery *et al.* [53] adopted the ETV approach to quantify Se in selenoprotein bands separated by SDS-PAGE. The gel was silver-stained and left to dry. Each lane was cut first into two equal strips in the direction of migration and then into bands (*ca.* 5 mm width and 1 mg dry mass). Analysis of a single gel lane with 10 bands could be accomplished in *ca.* 2 h. The approach included an external calibration with Te as internal standard and realised a detection limit of about 50 $ng \cdot g^{-1}$ (50 pg per gel strip). The method, developed using glutathione peroxidase as a test compound, was applied to fractionation of selenized-yeast materials [53, 54]. In the water and SDS-soluble protein extracts, several major bands were discerned in the mass range from lower than 14 kDa to higher than 94 kDa (Fig. 3). The distribution pattern was confirmed by gel filtration preparative chromatography [54].

LA linked to ICP-MS for gel analysis was first described in 1998 [13]. Table 1 presents a brief comparison of applications from the standpoint of electrophoresis platform, laser/ICP instrumentation and analytical performance. In the proof-of-principle study, 2-D crossed IEP on agarose gels was performed for the separation and detection of protein-bound Co species in human serum (artificially enriched with Co spike). Intensity distributions (Co) throughout the entire 2-D gel were realized by repetitive line rastering and this permitted construction of a distribution map for Co. Comparison of the Co distribution map with the protein distribution map obtained *via* Coomassie Brilliant Blue staining of molecular markers allowed identification of five main Co binding serum proteins (albumin, α_2-macroglobin, β_1-lipoprotein, α_1-lipoprotein, α_1-antitrypsin, and haptoglobin). Signal quantitation was achieved by casting gels enriched with increasing concentrations of Co and peak area response was linear with concentration. Of more immediate clinical significance was a speciation study directed at Pt anticancer drugs, cis- and carbo-platin [15]. In patients receiving

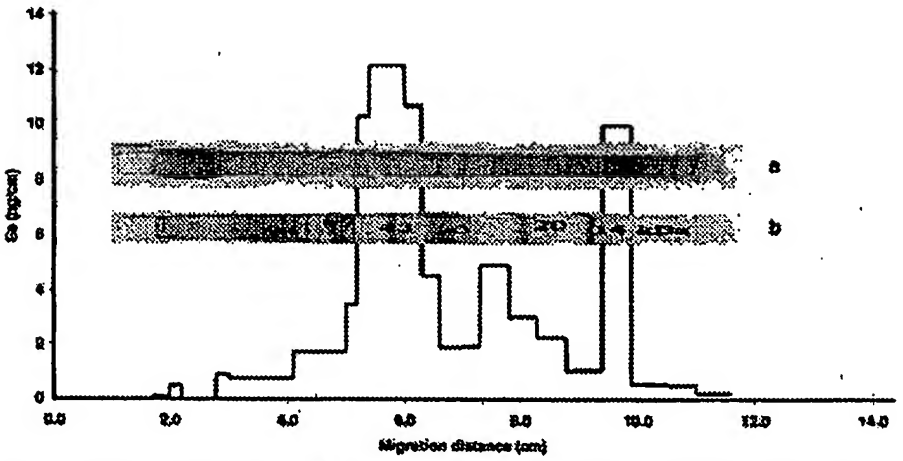

Figure 3. SDS-PAGE/ETV-ICP-MS (^{82}Se) profile for SDS-soluble fraction of selenised yeast. (a) Sample gel, (b) gel with molecular mass markers. Reproduced from [54].

Table 1. Metalloprotein studies based on LA-ICP-MS

Analyte/matrix	GE format	LA/ICP-MS	Limit of detection	Ref.
Co, serum	2-D IEP	Nd:YAG (1064 nm), quadrupole	8.2 $\mu g \cdot mL^{-1}$	[13]
Pt, serum	PAGE	Nd:YAG (266 nm), quadrupole	0.005 $\mu g \cdot mL^{-1}$	[15]
Pb, standard	SDS-PAGE	Nd:YAG (266 nm), quadrupole	–	[62]
Pt, *E. coli*	SDS-PAGE	Nd:YAG (266 nm), quadrupole	–	[55]
P, standard	SDS-PAGE	Nd:YAG (213 nm), quadrupole	–	[18]
Se, tissue	SDS-PAGE	Nd:YAG (213 nm), quadrupole-DRC	–	[22]
Zn, Cd and S, *E. coli*	PAGE	Nd:YAG (266 nm), quadrupole	–	[17]
P and S, tissue	2-DE	Nd:YAG (266 nm), sector field	0.6 and 150 $\mu g \cdot g^{-1}$	[20, 56]
P, standard	SDS-PAGE	Nd:YAG (266 nm), sector field	0.15 ng	[19]
Se, yeast	SDS-PAGE and 2-DE	ArF (193 nm), quadrupole-DRC	0.15 and 0.07 $\mu g \cdot g^{-1}$	[21, 61]
P, standard	SDS-PAGE	Nd:YAG (266 nm), quadrupole-DRC	0.6 $\mu g \cdot g^{-1}$	[59, 60]

such therapy concentrations in blood serum are elevated (typically 100–500 $\mu g \cdot L^{-1}$) and from a toxicity standpoint it is important to study the rate and extent of uptake of existing and new candidate drugs. As shown in Fig. 4 for both *in vitro* and *in vivo* experiments laser line rastering of gels (1-D PAGE) revealed extensive Pt complexation reflecting a high degree of reactivity for the Pt compounds with serum proteins. Analogous studies with standard proteins (albumin, transferrin, and α_2-macroglobin) were performed to aid interpretation.

In an attempt to discover protein drug targets from whole bacterial cell systems, *Escherichia coli* was treated with the Pt-based anticancer drug cisplatin [55]. Using LA-ICP-MS, the highest Pt level was found in the nonreducing SDS-PAGE band containing the outer membrane protein A (OmpA), implying a role for OmpA in cisplatin uptake. A 1-D PAGE approach has been used to characterize protein-bound Zn and Cd in *E. coli* cellular cytoplasmic fractions [17]. *E. coli* cells were cultured with or without the metal ions of interest. For an *E. coli* strain overexpressing the cyanobacterial metallothionein SmtA derived from *Synechococcus* as a positive control, induction of SmtA by Zn stress was observed and both Zn and S (as a protein marker) signals were significantly enhanced relative to the control (Fig. 5). When wild-type *E. coli* cells were grown in the presence of toxic but sublethal levels of metals, at least seven proteins with elevated metal (Zn or Cd) were detected and metal response was always coincident with S response showing the value of multi-

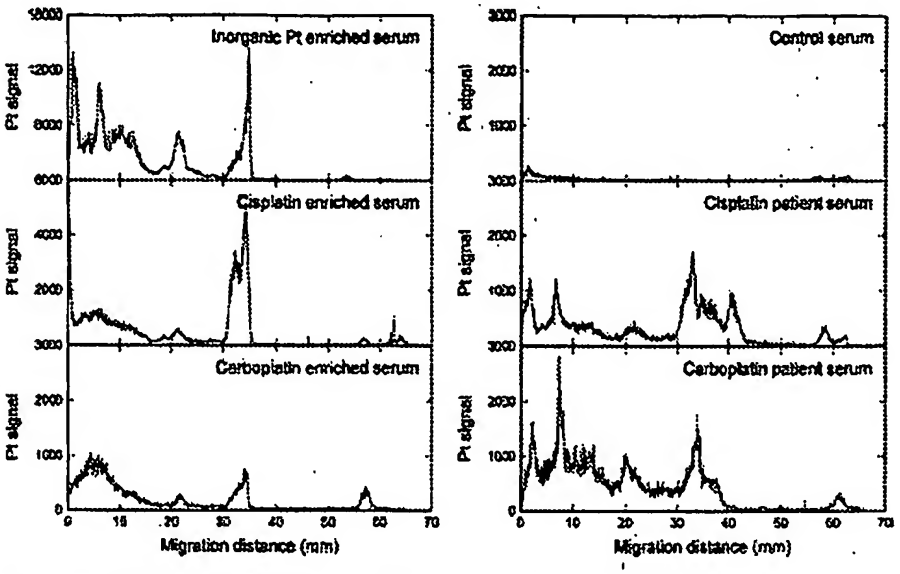

Figure 4. PAGE/LA-ICP-MS (^{195}Pt) profiles for blood serum *in vitro* and *in vivo*.

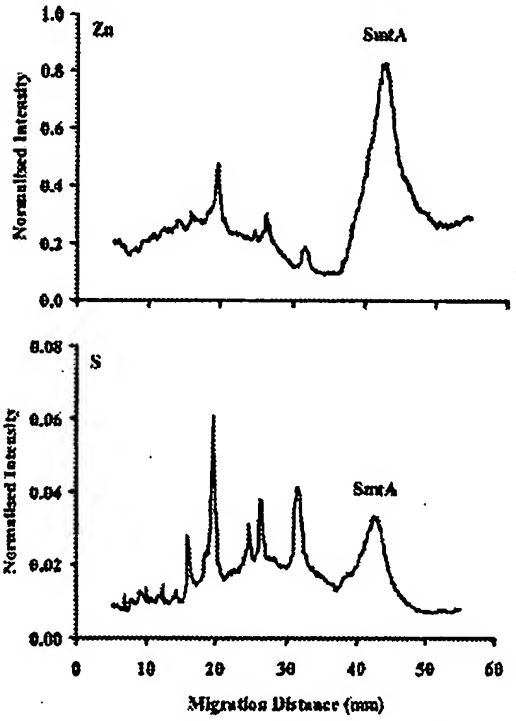

Figure 5. PAGE/LA-ICP-MS (^{66}Zn and ^{34}S) profiles for *E. coli* overexpressing metallothionein SmtA.

element detection for verification purposes. The Zn, Cd and S contents were quantified using metal-enriched gels with ^{13}C as internal standard.

The measurement of phosphoproteins by ICP-MS following GE or LC separation is of current interest. In a feasibility study, Marshall *et al.* [18] utilized ^{31}P to detect β-casein *via* LA of gels. Whereas direct measurement on SDS gels was hindered by background levels due to contamination, improved sensitivity was realized after transfer to a PVDF membrane. Subsequently, Becker *et al.* [20] utilized sector field ICP-MS for determination of P and S in proteins after LA of gels (2-DE). Calibration was established by introducing standard solutions *via* an ultrasonic nebulizer coupled to the LA chamber while a blank gel was ablated (introduced for gel analysis). High mass-resolution afforded by the sector instrument was required to eliminate spectral interference on $^{31}P^+$ due to molecular ions $^{15}N^{16}O^+$, $^{14}N^{17}O^+$ and $^{14}N^{15}O^1H^+$ derived from the nebulized aerosol. Cobalt was used as an internal standard and added to the gel after electrophoresis. In addition to β-casein, studies were also directed at phosphorylated human tau protein. The procedure was further applied to determination of P/S ratios in human brain proteins in an attempt to correlate data with Alzheimer's disease [56].

As already mentioned, background levels of P and S in gels were problematic. Wind *et al.* [19] successfully performed phosphoprotein quantitation *via* ^{31}P detection by LA-ICP-MS (sector) after SDS-PAGE separation and

PDVF membrane blotting transfer. A washing step with $Ga(NO_3)_3$ was found necessary to remove inorganic phosphate noncovalently bound to proteins before the membrane blot strips were dried. Recombinant bovine protein kinase A (PKA) catalytic subunit and a mixture of myoglobin, α-casein and reduced fibrinogen (Aα-, Bβ-, and γ-chains) were used to demonstrate that phosphoproteins were specifically recognized. A detection limit of 0.15 ng of phosphorus was estimated. It should be mentioned that the Wind group first demonstrated the potential of ICP-MS for phosphoprotein measurement in studies linking μLC to both quadrupole and sector field instrumentation [57, 58]. In an independent work, Bandura *et al.* [59, 60] employed ICP-MS with dynamic reaction cell (DRC) to permit phosphoprotein measurement *via* $^{31}P^{16}O^+$. The LA method yielded a detection limit of 0.6 $\mu g \cdot g^{-1}$ P following SDS-PAGE. An important aspect of this research [60] was a short clinical investigation where elevated P/S ratio values were found to be consistently higher for malignant tissue (colorectal cancer) *versus* normal tissue.

A study by Fan *et al.* [22] concerned Se speciation in Se-contaminated wildlife. Experiments based on SDS-PAGE/LA-ICP-MS involved protein extracts of an avocet embryo and a bass ovary collected from Se-rich waters and were designed to classify mechanisms of toxicity. For the two samples, eight and five distinct bands of selenoproteins were revealed, respectively, of which four bands (>100, 60, 50, and 48 kDa) derived from each sample category had comparable molecular weights. The ICP-MS (quadrupole) was equipped with DRC and used methane as reaction gas to eliminate the argon dimer (Ar_2^+) interference thus permitting use of the two most abundant Se isotopes (^{80}Se and ^{78}Se) for enhanced detection. Quantitation was based on using a selenoprotein standard for calibration. More recently, selenoproteins in red blood cell and yeast extracts were studied using 1-D SDS-PAGE and 2-DE, respectively [21]. Detection limits for ^{80}Se with CO as reaction gas and helium as transport gas (for LA aerosol) were 0.07 $\mu g \cdot g^{-1}$ for spot ablation (2-D) and 0.15 $\mu g \cdot g^{-1}$ for line rastering (1-D). Some ten yeast selenoproteins in the mass range 9–20 kDa were later characterized [61]. In an environmental application, Evans *et al.* [62] utilized SDS-PAGE with LA-ICP-MS to characterize Pb binding to various molecular size fractions of humic and fulvic acids. Further activity in this area is warranted.

5 Future developments

Significant advances have been achieved for the speciation of protein-bound trace elements by GE-atomic spectrometry. In particular, high-resolution high-sensitivity speciation measurement can be realized by judicious choice of the hyphenated analytical system. 1-D format is relatively convenient in terms of analysis time whereas a 2-D strategy although requiring additional sample handling steps is the benchmark for high-resolution work. Clearly 2-D approaches benefit from automated sample handling/processing technologies.

ICP-MS is a powerful multielement technique capable of high-throughput analysis. Sector field instrumentation offers highest sensitivity and good mass resolution but, as indicated in many studies, quadrupole mass analyzers including those equipped with reaction/collision cell technology are competitive in targeted applications. Relative advantages of laser ablation *versus* solution nebulization in the context of gel analysis have yet to be elaborated but it is likely that future hardware developments that yield impacts on method sensitivity, selectivity, and speed of analysis will accelerate interest in speciation research. One aspect of ICP-MS measurement yet to be fully exploited is the use of enriched stable isotopes for tracer investigations. Measurement of alternative isotopes can also help to avoid spectral and background interference problems.

In the future, comprehensive chemical speciation will increasingly rely on elemental and molecular mass spectrometric methods, principally electrospray ionization (ESI)- or matrix-assisted laser desorption/ionization (MALDI)-MS. This is well illustrated by recent studies on phosphoprotein [20] and selenoprotein [61] analyses and Pt protein binding studies [55]. It is clear that synergies and complementarities exist between separatory schemes and elemental/molecular mass spectrometries and that judicious combinations of GE, CE, and LC with dual elemental/molecular MS detection will play an increasingly important role in future metalloprotein research.

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